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A improved design of spiral tube assembly for separation of proteins by high-speed counter-current chromatography

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Abstract

A new spiral tube assembly was designed to improve the column capacity and partition efficiency for protein separation. This spiral tube assembly has greater column capacity than the original tubing because of an increase in radial grooves from 4 to 12 to accommodate more spiral layers and 12 narrow spots instead of 4 in each circular loop to interrupt the laminar flow that causes sample band broadening. Standard PTFE tubing (1.6 mm ID) and the modified flat-twisted tubing were used as the separation column. The performances of both assemblies were compared for separating 3 stable test proteins including cytochrome c, myoglobin, and lysozyme using a two phase aqueous-aqueous solvent system composed of polyethylene glycol 1000 (12.5% w/w) and dibasic potassium phosphate (12.5% w/w). All samples were run at 1, 2, 3, and 5 mL/min at both 800 RPM and 1000 RPM. The separation of these three protein samples produced high stationary phase retentions at 1, 2, and 3 mL/min, yet separated efficiently at 5 mL/min in 40 minutes. After comparing the separation efficiency in terms of the peak resolutions, theoretical plate numbers, and separation times, it was determined that the flat-twisted tubing was more effective in separating these protein samples. In order to validate the efficacy of this novel assembly, a mixture of five protein samples (cytochrome c, myoglobin, ovalbumin, lysozyme, and hemoglobin) were separated, under the optimal conditions established with these 3 protein samples, at 1 mL/min with a revolution speed of 1000 RPM. There were high stationary phase retentions of around 60%, with effective separations, demonstrating the efficiency of the flat-twisted spiral tube assembly. The separation time of 6 hours was a limitation but can potentially be shortened by improving the strength of the column that will permit an increase in revolution speed and flow rate. This novel spiral separation column will allow rapid and efficient separation of mixtures with high yield of the constituent components.

Keywords

Spiral tube assembly; flat-twisted tubing; high-speed countercurrent chromatography; protein separation; polymer phase system

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1. Introduction

Countercurrent chromatography (CCC) is a liquid-liquid partition chromatographic technique with a separation column free of solid support [1-6]. It eliminates irreversible adsorption of samples on the solid support used in liquid chromatography to yield high sample recovery. In this system the stationary liquid phase is retained in the column by either a stable force field [1,7,8] (hydrostatic system) or an Archimedean Screw effect generated by a combination of the coiled tube configuration and rotating force field (hydrodynamic system) [9]. Among various types of hydrodynamic CCC systems, high-speed CCC using a multilayer coil separation column has an efficient separation capability and it has been widely used for separation of natural products with moderately hydrophobic two-phase solvent systems such as hexane-ethyl acetate-methanol-water at various volume ratios [10, 11]. However, separations of polar compounds such as proteins and peptides have produced some problems in the retention of stationary phase to limit the application of the method. In order to challenge this problem, we have developed a new separation column with a spiral tube arrangement [12-15]. In this design the spiral pitch forms a centrifugal force gradient along the radial direction to move the heavier phase toward the periphery and the lighter phase in the opposite direction to enhance the retention of the stationary phase. This spiral tube assembly with 4 interwoven spiral tubes has been efficiently used for separation of peptides and proteins with polar two-phase solvent systems. One problem in the design of this original spiral tube assembly is that in the four radial transfer grooves, the transfer tube is inserted in every other spiral layer to limit the number of spiral layers accommodated in the separation column. In the improved spiral column design introduced in this paper, the layout of the radial grooves is modified in such a way that the number of radial transfer grooves is increased from 4 to 12 so that three spiral layers are accommodated between the transfer tubes to increase the number of spiral layers in a given column. In the present studies, the performance of this new spiral tube assembly is examined on two spiral columns: one made of standard 1.6 mm ID Teflon tubing and the other flat-twisted tubing made by extruding the same tubing through a narrow slit followed by twisting along its axis. Using these two columns, the separation was performed with a set of stable test proteins using an aqueous-aqueous polymer phase system composed of polyethylene glycol 1000 and dibasic potassium phosphate each at 12.5% (w/w) in distilled water. The performance of these two columns was compared in terms of theoretical plate number, peak resolution and retention of the stationary phase under the combination of various flow rates and revolution speeds.

2 Experimental

2.1. Apparatus

The CCC instrument used in the present studies is a type-J coil planet centrifuge purchased from P.C. Inc., Potomac, MD. USA. It holds a spiral tube assembly and its counterweight symmetrically on the rotary frame at a distance of 10 cm from the central axis of the centrifuge. The revolution speed is regulated with a speed controller (Bodine Electric Co., Boston, MA) from 0 – 1200 rpm while 800 and 1000 rpm were used in the present studies. The separation column was made by inserting PTFE (polytetrafluoroethylene) tubing into a

spiral tube support (STS) to form a spiral tube assembly. STS was made of Nylon with the laser sintering technique (CC Biotech LLC, Rockville, MD, USA). It is 17 cm diameter and 5 cm high disk with 4 interwoven spiral grooves each 3 mm wide and 4 cm deep with 12 radial transfer grooves to accommodate the connection tubing. Two spiral tube assemblies were made, one from a single piece of PTFE tubing of 1.6 mm I.D. (Zeus Industrial Products, Orangeburg, SC, USA) and the other from flat-twisted tubing made from the same tubing by extruding it through a narrow slit followed by twisting along its axis to form flat-twisted configuration [16]. The column was made by inserting the tubing into the spiral grooves using a pressing tool with 12 protrusions to compress the connection tubing into the radial grooves. The total 13 spiral layers were made from standard tubing at a total capacity of 135 ml and 10 spiral layers from the flat-twisted tubing with a total capacity of 90 ml. The column with the modified tubing has much less capacity in its narrow opening and less number of spiral layers in the limited column space due to difficulty of squashing the flat-twisted tubing in the radial grooves. A Shimadzu HPLC pump (Shimadzu Corp., Kyoto, Japan) was used to pump the solvent; a Uvicord IIs (LKB, Stockholm, Sweden) to monitor the effluent; and a strip-chart recorder (Farmacia, Sweden) to trace the chromatogram.

2.2. Reagents

Polyethylene glycol 1000 (PEG1000) was obtained from Merck, Darmstadt, Germany, and dibasic potassium phosphate, cytochrome c, myoglobin, ovalbumin lysozyme, and hemoglobin were all purchased from Sigma Chemicals, St. Louis, MO, USA.

2.3. Preparation of two-phase solvent system and sample solutions

The two-phase solvent system used in the present studies was prepared as follows: The stock solutions of PEG 1000 and dibasic potassium phosphate were prepared by dissolving each 25% (w/w) in deionized water in a separate bottle. Then an equal volume (200 mL) of each stock solution was mixed in a separatory funnel, and the upper and lower phases were separated shortly before use. Sample solution for the test separation was prepared by dissolving cytochrome c (1 mg), myoglobin, (10 mg) and lysozyme (10 mg) in 2.5 mL of the upper phase. Sample solution for a preparative separation was prepared by dissolving cytochrome c, (2.5 mg) myoglobin, (10 mg) ovalbumin, (15 mg) lysozyme, (25 mg), and hemoglobin (5 mg) in 2.5 mL of the upper phase.

2.4. Determination of partition coefficient (K) of protein samples

K values of each protein samples were measured as follows: in a test tube (13 mm × 100 mm) 2 mL of each phase of equilibrated two-phase solvent system was delivered to which a small amount of the sample was dissolved. The test tube was capped and the contents were thoroughly mixed with a vortex mixer. After the two phases were separated, 200 µL of each phase was mixed with 2 mL of distilled water to determine the absorbance at 280 nm with a spectrophotometer (Genesis 10 uv, Thermo Spectrometric, Rochester, New York, USA). The K values were computed by dividing the absorbance of the upper phase with that of the lower phase.

2.5. CCC separation procedure

Each separation was initiated by filling the entire column with the upper stationary phase. Then the lower mobile phase was eluted through the column from the internal spiral terminal at a given flow rate (1 – 5 mL/min) in a tail to head direction while the column was rotated at the desired speed (800 or 1000 rpm). After the hydrodynamic equilibrium was established in the column, the rotation and elution were temporarily stopped, and the sample solution was injected into the column to restart the run. The effluent from the outlet of the column was continuously monitored with a UV detector (LKB Uvicord IIs) at 280 nm and the chromatogram was traced with a strip-chart recorder (Pharmacia, Sweden). In order to improve the tracing of each chromatogram, the effluent from the outlet of the column was mixed with water at the inlet of the monitor using a dialysis tube (Spectrum, CA, USA) at a flow rate of 0.1-0.5 mL/min or 1/10 of the mobile phase.

2.6. Analysis of partition efficiency

Partition efficiency of each separation was evaluated in terms of theoretical plate number (N) and peak resolution (Rs) from the obtained chromatogram using the conventional formula:

$$N = (4R/W) \quad (1)$$

$$Rs = 2(R_2 - R_1) / (W_1 + W_2) \quad (2)$$

where R is the retention volume and W, the peak width in volume, and the subscripts 1 and 2 indicate peaks 1 and 2, respectively

3. Centrifugal force diagram of spiral undergoing type-J planetary motion

The centrifugal force acting on the spiral column was analyzed to draw the force distribution diagram as follows: An Archimedean spiral revolves around the centrifuge axis at ω and simultaneously rotates its own axis at the same angular velocity in the same direction (type-J planetary motion). Then we wish to analyze the centrifugal force vectors acting on the various points on the spiral. In Fig. 1A the center of the spiral (Os) is initially located on the x-axis and the arbitrary point P on the spiral is at the distance r from Os and at angle α from the x-axis. After the lapse of time t, the spiral is located at angle θ and the arbitrary point P is found at angle $(2\theta + \alpha)$ as shown in Fig. 1B.

The analysis of acceleration is initiated by expressing the location of point P by the following two equations:

$$x = R \cos \omega t + r \cos (2\omega t + \alpha) \quad (3)$$

$$y = R \sin \omega t + r \sin (2\omega t + \alpha) \quad (4)$$

where $\omega t = \theta$.

The acceleration acting on the point P is then expressed by the second derivatives of these two equations, i.e. :

$$d^2x/dt^2 = -R\omega^2 \cos \omega t - 4r\omega^2 \cos (2\omega t + \alpha) \quad (5)$$

$$d^2y/dt^2 = -R\omega^2 \sin \omega t - 4r\omega^2 \sin (2\omega t + \alpha) \quad (6)$$

Now we select one position of the spiral, the center of which is located on the x-axis. This reduces the equations 3 and 4 as follows:

$$d^2x/dt^2 = -R\omega^2 - 4r\omega^2 \cos \alpha \quad (7)$$

$$d^2y/dt^2 = -4r\omega^2 \sin \alpha \quad (8)$$

From Equations 5 and 6, the centrifugal force vector of every point on the spiral can be obtained by choosing the suitable value of angle α and radial length of r .

Fig. 2 shows the centrifugal force vectors on various points on the spiral. The strength of the vector acting on Os is $R\omega^2$ which is used as the unit strength to express the force strengths on other points.

The obtained force distribution diagram clearly illustrates that the two phases are separated in the loop away from the central axis of the centrifuge under a strong force field, while they are efficiently mixed in the loop near the central axis in the reduced force field to repeat the partition process at a high frequency.

4. Results and discussion

Figures 3 and 4 show the CCC chromatograms of test samples by the semi-preparative (2) PTFE tubing that was flattened and twisted, run at four different flow rates (1, 2, 3, and 5 mL/min). All flow rates were tested at both 800 and 1000 revolutions per minute (see Tables 1 and 2). Both assemblies provided similar resolutions, theoretical plate numbers, and retention of the stationary phase, with low retentions at 5 mL/min and high retentions at 1, 2, and 3 mL/min flow rates in this order. A clear recording was achieved with the dilution of the sample with water through a dialysis tube that was inserted before the inlet of the monitor. The best separation was observed at 1 mL/min at 1000 RPM, with the peak resolution and the theoretical plate numbers being the highest in both STS assemblies. One disadvantage, however, was the prolonged separation time, which was 6 hours for the standard STS assembly and 5.5 hours for the flat-twisted STS assembly both run at 1 mL/min. Increasing the flow rate decreased the separation time significantly but also decreased the peak resolution, while effective separation of the compounds was still achieved. In addition, increasing the revolution speed also increased the effectiveness of the separation of the compounds along with increasing the separation time. Both STS assemblies, the standard and the flat-twisted tubing, provided similar peak resolutions, theoretical plate numbers, and

retention of the stationary phase, but the flat-twisted tubing was more efficient with less separation times for lower flow rates (See Tables 1 and 2). To further demonstrate the performance of the flat-twisted spiral tube assembly, five protein samples of Cytochrome C (2.5 mg), Myoglobin (10 mg), Ovalbumin (15 mg), Lysozyme (25 mg) and Hemoglobin (5 mg) were separated at a flow rate of 1 mL/min at 1000 RPM (see Figure 5) where Elution-Extrusion CCC [17] was done after the elution of the ovalbumin peak to shorten the separation time. High stationary phase retention was achieved along with effective separations of all peaks. Due to a large peak resolution between myoglobin and lysozyme (Table 2), ovalbumin was completely resolved between these two peaks.

5. Conclusions

In the present study, efficient separation was provided by a novel STS assembly. The conventional multilayer coil separation column has a lower stationary phase retention, which limits the separation efficiency of the system especially for hydrophilic solvent systems. The advantage of the present spiral column design is that the column capacity is substantially increased for a given size of the spiral column by spreading the radial grooves from 4 to 12 so that the three spiral layers are accommodated between the transfer tube (see Figure 1B), whereas in the original spiral column [12] and also recently designed analytical column [18], each spiral layer was sandwiched between the radial transfer tubing that limited the column capacity. Further, the present design gives 12 narrow spots (instead of 4) in each circular loop to interrupt the laminar flow that results in sample band broadening. Separation could be further improved with the present spiral column design by increasing the revolution speed, but this is limited due to the durability of the column. We are planning to improve the strength of the column in the future in order to use higher RPMs to improve the effectiveness of the separation.

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Cross-sectional view through the radial transfer grooves

Figure 1.

Design of the novel spiral tube support: a) aerial view of the support; b) presentation of the cross-sectional view through the radial grooves for comparison between the conventional (right) and the present (left) designs

Figure 2. Force distribution diagram of the spiral column under type-J planetary motion: a) The x-y coordinate system for analysis of the centrifugal force on the spiral tube system; b) Patterns of distribution of centrifugal force vectors in the spiral tube system undergoing type-J planetary motion

Figure 3.
Representative chromatograms of the STS assembly with standard tubing at flow rates of 1,2, 3, and 5 mL/min compared at 800 and 1000 RPM

Figure 4. Chromatogram of the STS assembly with flat-twisted tubing at flow rates of 1,2,3, and 5 mL/min compared at 800 and 1000 RPM

Figure 5.

Representative chromatogram of the flat-twisted tubing STS assembly with 5 protein samples as a demonstration of the effectiveness of the flat-twisted tubing configuration. Experimental conditions: two-phase solvent system: PEG1000 and K₂HPO₄ each 12.5% (w/w); Flow rate: 1mL/min; mobile phase: lower phase, EECCC was started after elution of ovalbumin peak (4 hours); revolution: 1000 RPM; Column Capacity: 90 mL; Stationary Phase Retention: 60%; Sample: 2.5 mL upper phase containing cytochrome c (2.5 mg)(K = 0.025), myoglobin (10 mg) (K = 0.57), ovalbumin (15 mg)(K = 1.95), lysozyme (25 mg)(K = 2.27), hemoglobin (5 mg)(K = 10.6)

Table 1

Performance of standard STS Assembly

Flow Rate (mL/min)	RPM	TP (Cyt C/Myo/Lys) *	Rs (Pk 1-2/Pk 2-3) **	Time (hours)	Sf (%) ***
1	800	118/41/-	1.34/-	5	61
2	800	301/77/104	1.38/1.93	1.5	52
3	800	576/84/484	0.88/2.61	1.5	53
5	800	-/484/128	-/1.5	0.8	21
1	1000	361/201/47	2.53/2.02	6	68
2	1000	784/172/171	2.00/2.64	2.5	59
3	1000	484/114/123	1.25/2.11	1.5	45
5	1000	-/361/106	-/1.77	0.7	33

* TP: Theoretical Plate Number of Cytochrome C (1 mg)/Myoglobin (10 mg)/Lysozyme (10 mg)

** Rs: Peak resolution

*** Sf: Stationary phase retention

Table 2

Performance of flat-twisted STS Assembly

Flow Rate (mL/min)	RPM	TP (Cyt C/Myo/Lys) *	Rs (Pk 1-2/Pk 2-3) **	Time (hours)	Sf (%) ***
1	800	348/88/96	1.87/2.64	3.5	69
2	800	178/164/135	1.50/1.87	1.5	31
3	800	1600/144/100	1.20/1.71	1	33
5	800	-/196/400	-/2	0.5	19
1	1000	576/71/187	2.09/3.23	5.5	66
2	1000	256/104/153	1.6/2.42	3	59
3	1000	1024/215/104	1.5/2	0.8	38
5	1000	64/196/100	2.66/2.50	0.5	20

* TP: Theoretical Plate Number of Cytochrome C (1 mg)/Myoglobin (10 mg)/Lysozyme (10 mg)

** Rs: Peak resolution

*** Sf: Stationary phase retention